Int Aquat Res (2017) 9:71–80
DOI 10.1007/s40071-017-0158-1

ORIGINAL RESEARCH

Development of a prototype 16S rRNA gene-based microarray for monitoring planktonic actinobacteria in shrimp ponds

Jingjing Wang · Wei Zhao · Yajie Liang · Yifan Han · Xingbiao Wang · Xiaoxia Zhang · Xiaomei Ma · Demin Zhang · Zhiyong Huang

Received: 18 October 2016 / Accepted: 11 February 2017 / Published online: 22 February 2017

Abstract Many reports have shown that the composition of the bacterioplankton community can serve as a biological indicator to evaluate the occurrence of shrimp diseases. However, the distribution, diversity, and function of planktonic actinobacteria in shrimp ponds are still poorly understood. In this study, a prototype of a 16S rRNA gene-based taxonomic microarray was developed and evaluated for monitoring of planktonic actinobacteria in shrimp ponds. The prototype microarray is composed of 30 probes that target ten dominant families of planktonic actinobacteria found in shrimp ponds. The specificity of the actinobacterial microarray was validated by a set of control hybridizations with 16S rRNA genes clones. The prototype microarray was subsequently tested with two seawater samples from ponds with diseased shrimp populations (PDS) and ponds with healthy shrimp populations (PHS). The actinobacteria hybridization profiles revealed a lower abundance of Microbacteriaceae and a higher abundance of Mycobacteriaceae in PDS than in PHS. The changes in planktonic actinobacterial communities were validated by pyrosequencing data. These results support the utility of the microarray for monitoring planktonic actinobacteria in shrimp ponds and aquaculture environments.

Keywords Microarray · Planktonic actinobacteria · Shrimp ponds · 16S rRNA gene · Aquaculture environment

Introduction

Predicting the occurrence of shrimp disease outbreaks in aquaculture farms can be of considerable value to the long-term sustainable development of the industry (Leung and Tran 2000). Monitoring multiple geochemical factors has been widely employed to generate water quality indices for disease forecasting (Ferreira et al. 2011; Ma et al. 2012). However, in practice, it is generally difficult to set a threshold value for a specific abiotic parameter to evaluate the disease risk precisely (Xiong et al. 2014b). Thus, the application of only traditional physicochemical variables to assess shrimp health status may be inadequate (Rao et al. 2000). Recently, a number of studies have revealed that the bacterioplankton community can serve as a biological

J. Wang · W. Zhao · Y. Liang · Y. Han · X. Wang · X. Zhang · X. Ma · Z. Huang (✉)
Tianjin Key Laboratory of Industrial Biological Systems and Process Engineering, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, No. 32, West 7th Road, Tianjin Airport Economic Area, Tianjin 300308, China
e-mail: huang_zy@tib.cas.cn

D. Zhang
School of Marine Sciences, Ningbo University, Ningbo 315211, China

indicator to evaluate the occurrence of shrimp diseases (Xiong et al. 2014b; Zhang et al. 2014). Actinobacteria, which are a prolific source of secondary metabolites with antibacterial, immunosuppressive, and antitumor activity (Becerril-Espinosa et al. 2013), are widely distributed in aquatic environments, where they play important roles (Subramani and Aalbersberg 2012; Ward and Bora 2006). However, the distribution, diversity, and function of planktonic actinobacteria in shrimp ponds are still poorly understood.

Bacterioplankton communities can be estimated easily and rapidly using techniques, such as high-throughput sequencing and phylogenetic microarray (phylochip; Ma et al. 2016; Zhang et al. 2013). However, data analysis of high-throughput sequencing techniques requires highly trained specialists, which has restricted its application in large-scale biomonitoring efforts (Seong Woon et al. 2010). Less expensive and simple analyses using phylochips make this a powerful method for routine monitoring of selected target sequences using hundreds of samples on a large scale (Seong Woon et al. 2010). The most common target of the phylochip is the 16S rRNA gene because of its ubiquity in the prokaryotic kingdom and its large presence in databases, which enables access to almost all bacteria by PCR (Bruce et al. 1992; Woese 1987). Although a few oligonucleotide microarrays based on the 16S rRNA gene have been developed for monitoring bacteria in aquatic environments (Bianca et al. 2004; Desantis et al. 2007; Jörg et al. 2004), the full-scale application of microarray technology for aquaculture environmental monitoring is scarce. This is in part because bacterial communities in the aquatic environments are distinct from those in aquaculture environments (Xiong et al. 2015).

This study was conducted to develop a prototype 16S rRNA gene-based microarray for monitoring planktonic actinobacteria in shrimp ponds. To accomplish this, 30 probes (30 nt) targeting ten dominant families of planktonic actinobacteria in shrimp ponds were designed. This prototype microarray was validated using 16S rRNA gene clones and tested with seawater samples from shrimp ponds. The results were compared to those obtained from the pyrosequencing of the same samples.

Materials and methods

DNA samples

Six 16S rRNA gene clones belonging to different families of actinobacteria were collected from marine environments (Table 1). All sequences were then sequenced and submitted to the NCBI database. Plasmid DNA was extracted from the clones using the Axygen Plasmid Miniprep Kit (Axygen, Hangzhou, China).

On the sampling day, approximately 1 L of seawater samples was collected from ponds with diseased shrimp populations (PDS) and ponds with healthy shrimp populations (PHS) located in Zhanqi, Ningbo, eastern China (29°32′N, 121°31′E; Zhang et al. 2014). After pre-filtering through nylon mesh (100 μm pore size) and 0.2 μm polycarbonate membrane (Millipore), water genomic DNA was extracted using a Power Soil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocols. DNA was quantified with a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and stored at −80 °C until amplification.

Table 1 Clones of 16S rRNA gene used to validate the microarray

Clones	Similar organisms	Identity (%)	Group	Accession number	Environmental source
C1	*Ilumatobacter sp.*	98	Acidimicrobiaceae	KP688285	East China Sea
C2	Uncultured Iamiaceae	99	Iamiaceae	KP688286	East China Sea
C3	*Mycobacterium smegmatis*	99	Mycobacteriaceae	KP688287	East China Sea
C4	Uncultured Cellulomonadaceae	99	Cellulomonadaceae	KP688288	South China Sea
C5	*Microbacterium sp.*	96	Microbacteriaceae	KP688290	East China Sea
C6	*Micrococcus* sp.	99	Micrococcaceae	KP688292	South China Sea

PCR amplification of 16S rRNA gene and labeling

The pMD®19-T vector specific primers M13-47/RV-M (Zhang et al. 2008) and the universal bacterial primers 27F/1492R (Tanner et al. 1999) were used to amplify 16S rRNA genes from clones and seawater samples, respectively. The PCR reaction mixture (total volume 25 μL) contained 12.5 μL of Green PCR Master Mix (2×) (Thermo Fisher Scientific, Shanghai, China), 1 μL of each primer (2.5 μM), 1 μL of genomic DNA or 16S rRNA gene clone (~20 ng), and 9.5 μl of ddH_2O. PCR consisted of denaturation at 94 °C for 3 min, followed by 30 cycles of 1 min denaturation at 94 °C, 1 min annealing at 55 °C, 1 min elongation at 72 °C, and a final elongation step of 10 min at 72 °C. PCR products (5 μL) were denatured with 3 μL of random primers Cy3-NNN NNN NNN (Sangon BioTech, Shanghai, China) and 9 μL of ddH_2O at 95 °C for 3 min, then treated with an ice bath for 3 min. Next, 2.5 μL 5 × Klenow Buffer, 2.5 μL NTP (2.5 mM), and 1 μL Klenow enzyme (NEB, Beijing, China) were added to the mixture, followed by incubation at 37 °C for 90 min and 70 °C for 10 min (Schenk et al. 2000).

Oligonucleotide probe design and microarray manufacturing

The phylogenetic software package ARB (http://www.arb-home.de) under Ubuntu with the ARB 16S rRNA database (ssu_jan04_corr_opt.arb) was used to design probes (Ludwig et al. 2004). The parameters of the probe design function were set as follows: 30 nt, $40 < GC\% < 60$, position 1–10 000 in *E. coli*, max target out of group of 0, min group = 10–50%. All probes were tested with the probe match function of ARB against the total ARB 16S rRNA database. The best probes, which had a weighted mismatch (WMM) value of <2 with the targeted taxon and >2 with non-targets, were selected from the probe results window. The predicted melting temperature Tm (according to the nearest neighbor method) was calculated using Oligo7 (Molecular Biology Insights, West Cascade, CO, USA) with the default settings. The probes were chosen preferably when they had the same melting temperature (Tm), no hairpin, and a stable homoduplex. The specificity of the probes was then checked using BLAST searches of the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). Probes having higher similarity (>90%) with the non-target group were removed from the probe set.

Probes were designed to target planktonic actinobacteria at the family level based on their predictive accuracy (Xiong et al. 2014b). Of the 42 families recognized in actinobacteria, the probes targeted ten families (Acidimicrobiaceae, Iamiaceae, Mycobacteriaceae, Cellulomonadaceae, Intrasporangiaceae, Microbacteriaceae, Micrococcaceae, Nocardioidaceae, Propionibacteriaceae, and Nitriliruptoraceae) shown to be dominant in shrimp ponds in the previous studies (Xiong et al. 2014a). Three probes targeted the same family, complementing several unique regions of the 16S rRNA gene (Loy et al. 2002).

Probes were synthesized (Sangon BioTech, Shanghai, China) with a 5′C6-NH_2 group for covalent attachment onto aldehyde slides AL (CapitalBio, Beijing, China), then spotted onto slides using a SmartArrayer 48 (CapitalBio, Beijing, China).

Hybridization protocols

The labeled DNA (15 μL) was mixed with 5 μL of hybridization buffer (5× SSC, 0.02% SDS, 5% formamide), denatured for 5 min at 95 °C, and immediately placed on ice. The slides were then placed in a hybridization chamber (CapitalBio, Beijing, China) and covered with a Hybri-slip (CapitalBio, Beijing, China). The labeled DNA was transferred onto the slide through holes in the Hybri-slip. Hybridization was conducted at 50 °C for 2 h, after which the slides were immediately washed by shaking in 2× SSC containing 0.02% SDS at 50 °C for 4 min, fresh 0.2× SSC at 50 °C for 4 min and 95% ethanol at room temperature for 4 min, successively. Finally, the slides were quickly dried by centrifugation at 1500 rpm for 4 min (Sanguin et al. 2006).

Scanning and data analysis

The slides were scanned at 532 nm at a resolution of 10 μm using a NimbleGen MS200 scanner (Roche NimbleGen). Images were analyzed with the GenePix 4.01 software (Axon, Union City, CA, USA). The spot

quality was visually checked, and spots of poor quality were excluded from further analyses. A given spot was considered to provide strong positive hybridization if the spot pixels had an intensity two times higher than the median pixel intensity of the local background plus twice the standard deviation of the local background. A given probe was only considered to be truly hybridized when at least three of four replicate spots provided a strong positive hybridization (Sanguin et al. 2006). The pixel intensity of positive hybridization was then normalized relative to a mean hybridization signal observed from the set of positive control (PC) probes to provide the best array-to-array consistency, because PC probes were universal for every hybridized PCR amplicon (Liles et al. 2010).

Pyrosequencing analysis

An aliquot (50 ng) of DNA from seawater samples (PDS and PHS) was used as the template for amplification. The V1–V3 hyper variable region of bacterial 16S rRNA genes was amplified using the primer set 27F and 519R (Xiong et al. 2014a). The bacterial 16S rRNA gene products were sequencing using a Roche FLX 454 pyrosequencing machine (Roche Diagnostics Corporation, Branford, CT, USA). The sequencing reads were analyzed by the Quantitative Insights Into Microbial Ecology (QIIMEv1.5.0) workflow (Caporaso et al. 2010; Zhang et al. 2014).

Results and discussion

Probe design and microarray format

Probes can be designed at various taxonomic levels quickly and easily with the ARB software using 16S rRNA gene databases (Ludwig et al. 2004). In this study, probes were designed to target planktonic actinobacteria at the family level, as previous work showed that the bacterioplankton communities had a higher predictive accuracy of shrimp health and disease at family level than other taxa (Xiong et al. 2014b).

The specificity and sensitivity of probes varying from length among 9 and 70 nucleotides (nt) were evaluated. Short probes (25 nt) were significantly more specific than longer ones (35 nt), but the latter were more sensitive. Probes with a length of 30 nt are often selected as the best compromise between required specificity and sensitivity (Relogio et al. 2002). To maintain homogenous hybridization conditions, probes with identical melting temperatures and GC content should be selected (Sanguin et al. 2006). When these requirements could not be met, probes with suboptimal conditions were accepted to provide adequate phylogenetic coverage. Finally, 30 probes targeting ten families of actinobacteria were designed. The probes were 29–31 nucleotides long (80% of the probes were 30 nucleotides long), with a G + C content between 48.3 and 65.5% (average 57.2%). The melting temperature was between 72.8 and 83.3 °C (90% of probes fell within the 80 ± 5 °C temperature ranges; Table 2). As hybridizations can occur even in case of non-perfect matches (Kyselková et al. 2008; Loy et al. 2002), the specificity of probes was assessed using both Probe Match and BLAST.

A schematic diagram of the probe positions on the microarray is presented in Fig. 1. One probe has spotted as a positive hybridization control (PC), one as a negative hybridization control (NC), and one as a printing control (CK). PC targeted the V3 region of the 16S rRNA gene. The 30 polyT corresponding to the sequences not amplified during PCR was spotted as NC, and the 30 polyT labeled by HEX was spotted as CK. Each probe was printed in quadruplicate. The basic probe pattern on the microarrays consisted of two PC, six NC, and four CK, which could be used as the position reference and landmarks for image analysis. The 5′ end of each oligonucleotide probe was tailed with ten dTTP molecules (T-spacer) to increase the on-chip accessibility of spotted probes to target DNA.

Validation of probe set with pure cultures

The specificity of the individual probe was evaluated with six clones belonging to six families, respectively. Highly specific signals were observed, and all targets could be clearly differentiated by the signal patterns

Table 2 Oligonucleotide probes used in this study

Probe	Specificity	Sequence (5′–3′)	*L* (bp)	Tm (°C)	GC%
ID 1	Acidimicrobiaceae	CTTGGCAAGTCGGATGTGAAATCTCCAGGC	30	77.2	53.3
ID 2	Acidimicrobiaceae	GAGTCCGGTAGAGGATCGTGGAATTCCTGG	30	77.1	56.7
ID 3	Acidimicrobiaceae	GAGTGCGGTAGGGGAGCGGGGAATTCCTAG	30	80.6	63.3
ID 4	Iamiaceae	CGAAGACCGGGATAACCCTCCGAAAGGAGA	30	78.4	56.7
ID 5	Iamiaceae	TGACTTGAGTCCGGTAGGGGAGCACGGAA	29	80.1	58.6
ID 6	Iamiaceae	GACACCGCGAGGTTGAGCGAATCCCACAAA	30	80.1	58.6
ID 7	Mycobacteriaceae	ACAGCTTAACTGTGGGCGTGCGGGCGATAC	30	81.4	58.6
ID 8	Mycobacteriaceae	TACTAGGTGTGGGTTTCCTTCCTTGGGATC	30	75.2	48.3
ID 9	Mycobacteriaceae	GTTCCCTTGTGGCCTGTGTGCAGGTGGTG	29	81.0	62.1
ID 10	Cellulomonadaceae	CCGGAAAAGTGCAGAGATGTGCTCCCCGTAA	31	80.0	58.6
ID 11	Cellulomonadaceae	ACCTCAGGCTCAACCTGGGGCTTGCAGTGGG	31	81.8	62.1
ID 12	Cellulomonadaceae	GTACACCGACCTTGCGGGGCAACCATCTCT	30	80.3	62.1
ID 13	Intrasporangiaceae	ATCCGGGGCTCAACCCCGGACTTGCAGTGG	30	83.3	65.5
ID 14	Intrasporangiaceae	AATCCGGGGCTCAACCCCGGACTTGCAGTG	30	83.2	63.3
ID 15	Intrasporangiaceae	GACATACACCGGACACATCCAGAGATGGGTG	31	77.2	53.3
ID 16	Microbacteriaceae	CAGTATCCCATGAGTTCCCACCATTACGTG	30	74.7	50.0
ID 17	Microbacteriaceae	TAGATACCCTGGTAGTCCACCCCGTAAACG	30	76.4	50.0
ID 18	Microbacteriaceae	CAGAGTCAAGGGCAGATTGCTCACGTGTTA	30	76.3	50.0
ID 19	Micrococcaceae	CTGTCGTGAAAGTCCGGGGCTTAACCCCG	29	80.1	62.1
ID 20	Micrococcaceae	CACACAAGGTGGTTAGGCCATCGGCTTCGG	30	79.0	58.6
ID 21	Micrococcaceae	GTCCGAGGCTCAACCTCGGATCTGCGGTGG	30	81.3	65.5
ID 22	Nocardioidaceae	CACCACCTGTACACCAGTATCAAAGAGACC	30	72.8	48.3
ID 23	Nocardioidaceae	CGAAGGCGGTTCTCTGGGCATTACCTGACG	30	78.4	58.6
ID 24	Nocardioidaceae	AATCCGTGGAAAGGACCCCACACCTAGCGC	30	79.6	58.6
ID 25	Propionibacteriaceae	TGCTTTCGATACGGGTTGACTTGAGGAAGG	30	74.7	48.3
ID 26	Propionibacteriaceae	AGCTCGTAGGTGGTTGATTGCGTCGGAAGT	30	78.0	55.2
ID 27	Propionibacteriaceae	TACAAAGAGTTGCGAGCCTGTGAGGGTGAG	30	77.4	51.7
ID 28	Nitriliruptoraceae	CCGTAGAGATGCGGTGGGTTCGTCCGTGCT	30	81.5	65.5
ID 29	Nitriliruptoraceae	CATAGGGTCCGAGCGTTGTCCGGAATCATT	30	77.8	55.2
ID 30	Nitriliruptoraceae	ACCTCCAGAAGAAGGACCGGCCAACTATGT	30	77.5	55.2
PC	Positive control	ACTCCTACGGGAGGCAGCAGTGGGGAATAT	30	80.0	58.6
NC	Negative control	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT	30	58.6	0
CK	Printing control	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-HEX	30	58.6	0

obtained (Fig. 2). Each of the expected-positive groups produced a hybridization signal with each of the positive control rRNAs. None of these probes showed false-negative or false-positive signals.

The full-scale application of 16S rRNA-based taxonomic microarray relies on a highly specific probe set (He et al. 2004). Identifications can be made with a high degree of confidence using an intelligent combination of several probes (Loy et al. 2005). Three probes that targeted separate signature sites of one family were constructed in our study. If they bound to the same colonies, or to the same fragment or fraction of DNA, the possibility of false positives is virtually eliminated. For example, in the hybridization results, two of three probes of Iamiaceae (ID 5 and ID 6) and Microbacteriaceae (ID 16 and ID 17) produced hybridization signals upon hybridization with clone C2 and clone C5, respectively (Fig. 2). The differences in the hybridization signals for expected-positive features between different rRNA gene sequences (e.g., clone 1 vs. clone 3) might be a consequence of different probe hybridization kinetics (Liles et al. 2010).

CK	CK	CK	CK	NC	NC	NC	NC	CK	CK	CK	CK
NC	NC	NC	NC	PC	PC	PC	PC	NC	NC	NC	NC
ID-1	ID-1	ID-1	ID-1	ID-2	ID-2	ID-2	ID-2	ID-3	ID-3	ID-3	ID-3
ID-4	ID-4	ID-4	ID-4	ID-5	ID-5	ID-5	ID-5	ID-6	ID-6	ID-6	ID-6
ID-7	ID-7	ID-7	ID-7	ID-8	ID-8	ID-8	ID-8	ID-9	ID-9	ID-9	ID-9
ID-10	ID-10	ID-10	ID-10	ID-11	ID-11	ID-11	ID-11	ID-12	ID-12	ID-12	ID-12
ID-13	ID-13	ID-13	ID-13	ID-14	ID-14	ID-14	ID-14	ID-15	ID-15	ID-15	ID-15
ID-16	ID-16	ID-16	ID-16	ID-17	ID-17	ID-17	ID-17	ID-18	ID-18	ID-18	ID-18
ID-19	ID-19	ID-19	ID-19	ID-20	ID-20	ID-20	ID-20	ID-21	ID-21	ID-21	ID-21
ID-22	ID-22	ID-22	ID-22	ID-23	ID-23	ID-23	ID-23	ID-24	ID-24	ID-24	ID-24
ID-25	ID-25	ID-25	ID-25	ID-26	ID-26	ID-26	ID-26	ID-27	ID-27	ID-27	ID-27
ID-28	ID-28	ID-28	ID-28	ID-29	ID-29	ID-29	ID-29	ID-30	ID-30	ID-30	ID-30
NC	NC	NC	NC	PC	PC	PC	PC	NC	NC	NC	NC
CK	CK	CK	CK	NC	NC	NC	NC	CK	CK	CK	CK

Fig. 1 Detailed design of a single array with exact positions for each probe (*CK* printing control, *PC* positive hybridization control, *NC* negative hybridization control, *ID-1–ID-30* probes targeting ten families of planktonic actinobacteria)

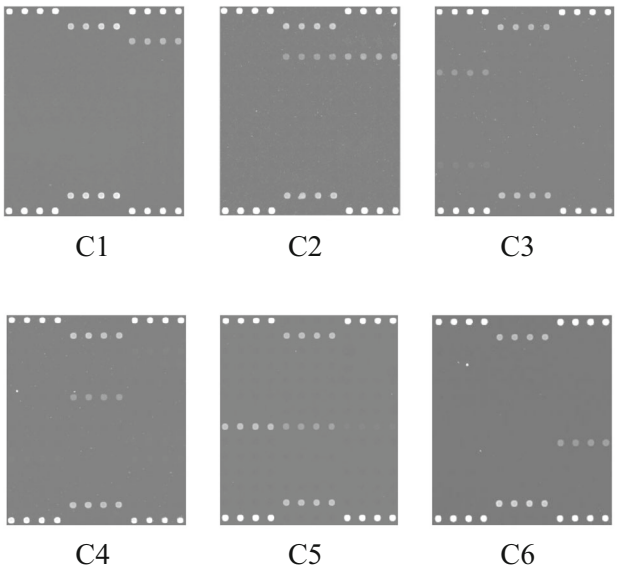

Fig. 2 Hybridization results of six reference clones. **C1**, *Ilumatobacter* sp. (Acidimicrobiaceae); **C2**, Uncultured Iamiaceae (Iamiaceae); **C3**, *Mycobacterium smegmatis* (Mycobacteriaceae); **C4**, Uncultured Cellulomonadaceae (Cellulomonadaceae); **C5**, *Microbacterium* sp. (Microbacteriaceae); **C6**, *Micrococcus* sp. (Micrococcaceae)

Hybridization of water samples

Two water samples from ponds with diseased shrimp populations (PDS) and ponds with healthy shrimp populations (PHS) were assessed by the actinobacterial microarray. Three of thirty probes produced hybridization signals upon hybridization with seawater samples from PHS. There was no hybridization signal produced by other probes. All three probes (ID16, ID17, and ID18) targeting Microbacteriaceae produced strong signals. ID16 (102%) generated the highest signals than others. ID 17 (51%) produced higher signals than ID 18 (8%). The hybridization patterns of the microarray indicated that Microbacteriaceae was present in PHS (Fig. 3a).

Four of the 30 probes generated signals upon hybridization with seawater samples from PDS (Fig. 3b). There was no hybridization signal produced by other probes. Of these four probes, three (ID16, ID17, and ID18) targeted Microbacteriaceae and one (ID7) targeted to Mycobacteriaceae. The signals of ID 7, ID 16, ID 17, and ID 18 were 35, 53, 19, and 19%, respectively. The hybridization patterns of the microarray indicated

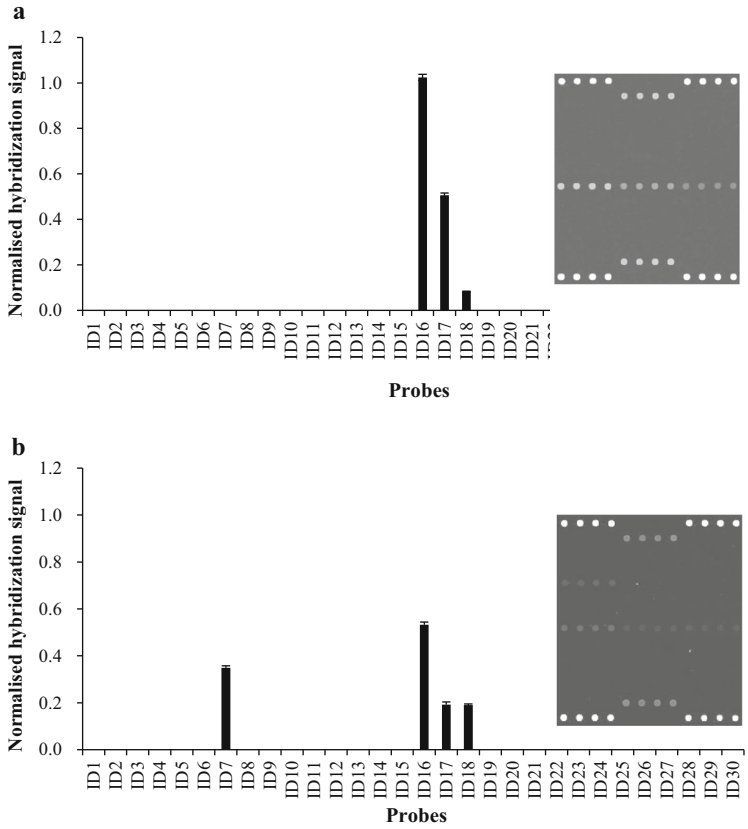

Fig. 3 Microarray analyses of seawater samples. **a** Hybridization of seawater samples from ponds with healthy shrimp populations (PHS). **b** Hybridization of seawater samples from ponds with diseased shrimp populations (PDS)

the presence of Microbacteriaceae and Mycobacteriaceae in PDS. However, the signal intensity of Microbacteriaceae in PDS was much lower than that in PHS.

It was of interest to compare the results of the actinobacterial community in healthy and diseased shrimp ponds, since the composition of bacterial communities in seawater can be altered by shrimps (Sombatjinda et al. 2011). The microarray hybridizations from seawater samples of PDS and PHS revealed some similarities, including the presence of the Microbacteriaceae. Interestingly, there was an apparent decrease in the hybridization signal for Microbacteriaceae and an apparent increase in the hybridization signal for Mycobacteriaceae in PDS compared with PHS. It should be noted that the probe hybridization signals could not be used to characterize the quantitative abundance of any group in an environmental sample due to potential kinetic differences between individual probes (Liles et al. 2010). However, if a comparison was conducted in the same set of probes with different environmental samples, a shift in probe hybridization strength from one sample to another could indicate a real shift in bacterial relative abundance (Liles et al. 2010). Mycobacteriaceae includes pathogens known to cause serious diseases in mammals. For example, *Mycobacterium marinum* was the causative agent of fish tank granuloma in humans (Rahman et al. 2014). However, no relationships between Microbacteriaceae or Mycobacteriaceae and shrimp disease were reported. The dynamics of actinobacterial communities may serve as an indicator of the health status of the shrimp (Berry et al. 2012).

Pyrosequencing results of water samples

The same DNA samples obtained from PHS and PDS were analyzed by pyrosequencing (Fig. 4). The results showed that the relative abundance of actinobacteria was 22.05% and that of Microbacteriaceae was 21.47% in PHS. Acidimicrobiaceae (0.07%), Acidimicrobiales_uncultured (0.04%), Candidatus_Microthrix (0.18%),

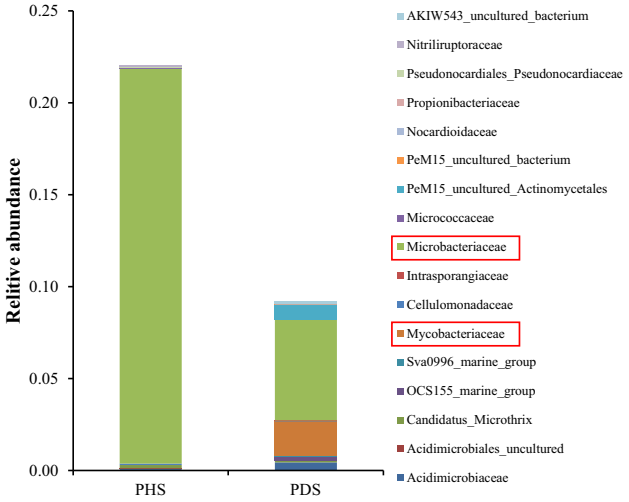

Fig. 4 Relative abundance of actinobacteria detected by Pyrosequencing (*PHS* seawater samples from ponds with healthy shrimp populations, *PDS* seawater samples from ponds with diseased shrimp populations)

Cellulomonadaceae (0.07%), Micrococcaceae (0.04%), Nocardioidaceae (0.02%), Propionibacteriaceae (0.02%), Pseudonocardiaceae (0.04%), and Nitriliruptoraceae (0.09%) had low-relative abundance in PHS. Overall, Microbacteriaceae accounted for 97.37% of actinobacteria and was a dominant actinobacterial family in PHS. Mycobacteriaceae was not detected in PHS.

Pyrosequencing of the PCR amplicons showed that relative abundance of actinobacteria was 9.17%, while that of Microbacteriaceae and Mycobacteriaceae was 5.49% and 1.88% in PDS, respectively. Acidimicrobiaceae (0.43%), Candidatus_Microthrix (0.13%), OCS155_marine_group (0.18%), Sva0996_marine_group (0.05%), Cellulomonadaceae (0.03%), Intrasporangiaceae (0.03%), PeM15_uncultured_Actinomycetales (0.79%), PeM15_uncultured_bacterium (0.03%), Nocardioidaceae (0.05%) and AKIW543_uncultured_bacterium (0.10%) had low-relative abundance in PDS. Microbacteriaceae (59.87% of actinobacteria), and Mycobacteriaceae (20.50% of actinobacteria) were dominant actinobacterial families in PDS.

The pyrosequencing results also indicated that Microbacteriaceae was present at high levels in both samples. The relative abundance of Microbacteriaceae was higher while that of Mycobacteriaceae was lower in PHS than in PDS. Therefore, the power of the microarray for predicting changes in actinobacterial communities in shrimp ponds was supported by the pyrosequencing analysis. Although high-throughput (or 'next-generation') sequencing can detect low-relative-abundance microorganisms in the environment (Andersson et al. 2010; Roesch et al. 2007), its high cost and complex analysis hinder its large-scale application to monitoring bacterial communities (Kircher and Kelso 2010; Seong Woon et al. 2010). The large parallel nature of phylogenetic microarray hybridizations enables rapid identification of bacterial groups and reflection of their changes in the environment (Brodie et al. 2006; Huyghe et al. 2008). Our result showed that actinobacterial microarray can detect high-relative-abundance actinobacteria and reflect their changes in health as well as in shrimp pond conditions. These findings indicated that the actinobacterial microarray has the potential for wide application to the monitoring of changes in actinobacterial communities in aquaculture environments. Future studies will expand beyond actinobacteria to encompass a broader diversity of bacteria. Eventually, this technique may be applied to monitor many microorganisms in the aquaculture environment.

Conclusions

We developed a 16S rRNA gene-based microarray that contained 30 probes targeting 10 dominant families of planktonic actinobacteria in shrimp ponds. The specificity of probes was validated by 16S rRNA genes clones. The availability of the microarray was tested using seawater samples from shrimp ponds, and the results were validated by pyrosequencing. Overall, the results indicated that the actinobacterial microarray might be widely

applicable to the monitoring of changes in planktonic actinobacterial communities in aquaculture environments.

Acknowledgements This work was financially supported by the Tianjin science and technology plan Project (15ZCZDSF00620), National High Technology Research and Development Program of China (863 Program, 2012AA092000) and Open Fund of Zhejiang Provincial Top Key Discipline of Aquaculture in Ningbo University (Grant No. xkzsc10). We thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.

References

Andersson AF, Riemann L, Bertilsson S (2010) Pyrosequencing reveals contrasting seasonal dynamics of taxa within Baltic Sea bacterioplankton communities. ISME J 4:171–181. doi:10.1038/ismej.2009.108

Becerril-Espinosa A, Freel KC, Jensen PR, Soria-Mercado IE (2013) Marine Actinobacteria from the Gulf of California: diversity, abundance and secondary metabolite biosynthetic potential. Antonie Van Leeuwenhoek 103:809–819

Berry D, Schwab C, Milinovich G, Reichert J, Ben Mahfoudh K, Decker T, Engel M, Hai B, Hainzl E, Heider S, Kenner L, Muller M, Rauch I, Strobl B, Wagner M, Schleper C, Urich T, Loy A (2012) Phylotype-level 16S rRNA analysis reveals new bacterial indicators of health state in acute murine colitis. ISME J 6:2091–2106. doi:10.1038/ismej.2012.39

Bianca C, Ermanno R, Andrea F, Kaarina S, Pirjo R, Anne R, Maria Angela M, Stefano V, Annick W, Christophe B (2004) Development of a universal microarray based on the ligation detection reaction and 16S rRNA gene polymorphism to target diversity of cyanobacteria. Appl Environ Microbiol 70:7161–7167

Brodie EL, DeSantis TZ, Joyner DC, Baek SM, Larsen JT, Andersen GL, Hazen TC, Richardson PM, Herman DJ, Tokunaga TK, Wan JMM, Firestone MK (2006) Application of a high-density oligonucleotide microarray approach to study bacterial population dynamics during uranium reduction and reoxidation. Appl Environ Microbiol 72:6288–6298. doi:10.1128/aem.00246-06

Bruce KD, Hiorns WD, Hobman JL, Osborn AM, Strike P, Ritchie DA (1992) Amplification of DNA from native populations of soil bacteria by using the polymerase chanin-reaction. Appl Environ Microbiol 58:3413–3416

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Tumbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7:335–336. doi:10.1038/nmeth.f.303

Desantis TZ, Brodie EL, Moberg JP, Zubieta IX, Piceno YM, Andersen GL (2007) High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment. Microb Ecol 53:371–383

Ferreira NC, Bonetti C, Seiffert WQ (2011) Hydrological and water quality indices as management tools in marine shrimp culture. Aquaculture 318:425–433

He Z, Wu L, Li X, Fields M, Zhou JZ (2004) Empirical establishment of oligonucleotide probe design criteriausing perfect match and mismatch probes and artificial targets. Appl Environ Microbiol 71:3753–3760

Huyghe A, Francois P, Charbonnier Y, Tangomo-Bento M, Bonetti EJ, Paster BJ, Bolivar I, Baratti-Mayer D, Pittet D, Schrenzel J, Geneva Study Grp N (2008) Novel microarray design strategy to study complex bacterial communities. Appl Environ Microbiol 74:1876–1885. doi:10.1128/aem.01722-07

Jörg P, Lau SCK, Jakob P, Rudolf A, Frank Oliver GC (2004) Application and validation of DNA microarrays for the 16S rRNA-based analysis of marine bacterioplankton. Environ Microbiol 6:638–645

Kircher M, Kelso J (2010) High-throughput DNA sequencing-concepts and limitations. BioEssays 32:524–536. doi:10.1002/bies.200900181

Kyselková M, Kopecký J, Felföldi T, Čermák L, Omelka M, Grundmann GL, Moënne-Loccoz Y, Ságová-Marečková M (2008) Development of a 16S rRNA gene-based prototype microarray for the detection of selected actinomycetes genera. Antonie Van Leeuwenhoek 94:439–453

Leung PS, Tran LT (2000) Predicting shrimp disease occurrence: artificial neural networks vs. logistic regression. Aquaculture 187:35–49

Liles MR, Turkmen O, Manske BF, Zhang M, Rouillard JM, George I, Balser T, Billor N, Goodman RM (2010) A phylogenetic microarray targeting 16S rRNA genes from the bacterial division Acidobacteria reveals a lineage-specific distribution in a soil clay fraction. Soil Biol Biochem 42:739–747

Loy A, Lehner A, Lee N, Adamczyk J, Meier H, Ernst J, Schleifer K-H, Wagner M (2002) Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. Appl Environ Microbiol 68:5064–5081

Loy A, Schulz C, Lücker S, Schöpfer-Wendels A, Stoecker K, Baranyi C, Lehner A, Wagner M (2005) 16S rRNA gene-based oligonucleotide microarray for environmental monitoring of the betaproteobacterial order "Rhodocyclales". Appl Environ Microbiol 71:1373–1386

Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar Buchner A, Lai T, Steppi S, Jobb G, Forster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, Konig A, Liss T, Lussmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A, Schleifer KH (2004) ARB: a software environment for sequence data. Nucleic Acids Res 32:1363–1371. doi:10.1093/nar/gkh293

Ma Z, Song X, Wan R, Gao L (2012) A modied water quality index for intensive shrimp ponds of *Litopenaeus vannamei*. Ecol Indic 24:287–293

Ma L, Mao G, Liu J, Gao G, Zou C, Bartlam MG, Wang Y (2016) Spatial-temporal changes of bacterioplankton community along an exhorheic river. Front Microbiol 7:1–12

Rahman SA, Singh Y, Kohli S, Ahmad J, Ehtesham NZ, Tyagi AK, Hasnain SE (2014) Comparative analyses of nonpathogenic, opportunistic, and totally pathogenic Mycobacteria reveal genomic and biochemical variabilities and highlight the survival attributes of *Mycobacterium tuberculosis*. Mbio 5:1–9. doi:10.1128/mBio.02343-14

Rao PSS, Karunasagar I, Otta SK, Karunasagar I (2000) Incidence of bacteria involved in nitrogen and sulphur cycles in tropical shrimp culture ponds. Aquac Int 8:463–472. doi:10.1023/a:1009250004999

Relogio A, Schwager C, Richter A, Ansorge W, Valcarcel J (2002) Optimization of oligonucleotide-based DNA microarrays. Nucleic Acids Res 30:1–10. doi:10.1093/nar/30.11.e51

Roesch LF, Fulthorpe RR, Riva A, Casella G, Hadwin AKM, Kent AD, Daroub SH, Camargo FAO, Farmerie WG, Triplett EW (2007) Pyrosequencing enumerates and contrasts soil microbial diversity. ISME J 1:283–290. doi:10.1038/ismej.2007.53

Sanguin H, Herrera A, Oger-Desfeux C, Dechesne A, Simonet P, Navarro E, Vogel TM, Moenne-Loccoz Y, Nesme X, Grundmann GL (2006) Development and validation of a prototype 16S rRNA-based taxonomic microarray for Alphaproteobacteria. Environ Microbiol 8:289–307. doi:10.1111/j.1462-2920.2005.00895.x

Schenk PM, Kazan K, Wilson I, Anderson JP, Richmond T, Somerville SC, Manners JM (2000) Coordinated plant defense responses in Arabidopsis revealed by microarray analysis. PNAS 97:11655–11660. doi:10.1073/pnas.97.21.11655

Seong Woon R, Abell GCJ, Kyoung-Ho K, Young-Do N, Jin-Woo B (2010) Comparing microarrays and next-generation sequencing technologies for microbial ecology research. Trends Biotechnol 28:291–299

Sombatjinda S, Boonapatcharoen N, Ruengjitchatchawalya M, Wantawin C (2011) Dynamics of microbial communities in an earthen shrimp pond during the shrimp growing period. Environ Nat Resour Res 1:171–180

Subramani R, Aalbersberg W (2012) Marine actinomycetes: an ongoing source of novel bioactive metabolites. Microbiol Res 167:571–580

Tanner MA, Shoskes D, Shahed A, Pace NR (1999) Prevalence of corynebacterial 16S rRNA sequences in patients with bacterial and "nonbacterial" prostatitis. J Clin Microbiol 37:1863–1870

Ward AC, Bora N (2006) Diversity and biogeography of marine actinobacteria. Curr Opin Microbiol 9:279–286

Woese CR (1987) Bacterial evolution. Microbiol Rev 51:221–271

Xiong J, Zhu J, Wang K, Wang X, Ye X, Liu L, Zhao Q, Hou M, Qiuqian L, Zhang D (2014a) The temporal scaling of bacterioplankton composition: high turnover and predictability during shrimp cultivation. Microb Ecol 67:256–264

Xiong J, Zhu J, Zhang D (2014b) The application of bacterial indicator phylotypes to predict shrimp health status. Appl Microbiol Biotechnol 98:8291–8299

Xiong J, Chen H, Hu C, Ye X, Kong D, Zhang D (2015) Evidence of bacterioplankton community adaptation in response to long-term mariculture disturbance. Sci Rep 5:1–11

Zhang Y, Wang C, Wang Z, Ma D, Pan S, Zhu S, Li F, Wang B (2008) Construction of double suicide genes system controlled by MDR1 promoter with targeted expression in drug-resistant glioma cells. J Neuro Oncol 86:3–11. doi:10.1007/s11060-007-9431-2

Zhang R, Wu Q, Piceno YM, Desantis TZ, Saunders FM, Andersen GL, Liu WT (2013) Diversity of bacterioplankton in contrasting Tibetan lakes revealed by high-density microarray and clone library analysis. FEMS Microbiol Ecol 86:277–287

Zhang D, Wang X, Xiong J, Zhu J, Wang Y, Zhao Q, Chen H, Guo A, Wu J, Dai H (2014) Bacterioplankton assemblages as biological indicators of shrimp health status. Ecol Indic 38:218–224